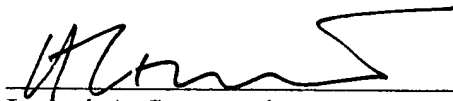Isabel Leonard
Medical and Technical Translation
5 Hearn Street, Watertown, MA 02472-1502, USA
Phone 617-661-3273 Fax 617-441-0036
e-mail: isabelleonard@comcast.net

File 7605

VERIFICATION OF TRANSLATION

I hereby declare and state that I am knowledgeable of each of the
German and English languages and that I made and reviewed the attached
translation of the PCT application entitled "Device and method for detecting
cellular processes by means of luminescence measurements" from the
German language into the English language, and that I believe my attached
translation to be accurate, true, and correct to the best of my knowledge and
ability.

Date: May 20, 2004

Isabel A. Leonard
Translator

Description

Device and method for detecting cellular processes by means of luminescence
measurements

The present invention relates to a method and a device for location-
specific detection of a luminescence event or for detection of a luminescence
signal in, at, or in the immediate vicinity of a cell to be analyzed.

A device according to the species for optical examination of a cell is
known from EP 0 881 490 A2. This device has a substrate, particularly a
semiconductor substrate, with a surface suitable for accepting cells, a number of
photodetectors formed below the surface, and multiple light sources above the
surface. The detectors arranged in a matrix pick up the light emitted by the light
sources, which brings about various light intensities at various detectors, which
are governed by a cell placed on the surface, from which information on the
geometry of the cell can be obtained.

For some applications, for example in pharmaceutical research, it is
particularly relevant to be able to investigate the behavior of cells in response to
a chemical or biochemical stimulus. Investigating metabolic processes in living
cells is particularly useful since for example the influence of a new potential drug
can thereby be observed. One measurement frequently performed in this
connection is intracellular calcium determination by calcium-sensitive dyes, Fura
II for example. A.L. Miller, E. Karplus, L.F. Jaffe: "Coelenterate Imaging
[Ca2+]$_i$ with Aequorin Using a Photon Imaging Detector," Meth Cell Bio. 40,
305 (1994) teaches the loading of cells, for example by osmotic shock treatment,
with a bioluminescent substance, for example aequorin. Intracellular calcium

signals as responses to chemical excitation are made visible due to the bioluminescence of aequorin.

The goal of the present invention is to provide a compact and easily realizable device and a method for detecting cellular processes by luminescence measurements using chemical or biochemical stimuli.

This goal is achieved by a device according to the features of Claim 1 and a method according to the features of Claim 16. Advantageous embodiments of the invention are the subjects of the dependent claims.

The device according to the invention comprises a carrier element with a surface prepared for direct or indirect coupling of cells, at least one optical detector to receive a luminescence signal, said detector being integrated into the carrier element below the surface, a cover covering the surface to form a cavity, said cover having an inlet opening and an outlet opening, and an excitation source connected to the inlet opening.

The excitation source constitutes a reservoir for a chemical or biological substance that for example influences the metabolism of the cell, with metabolic processes being made visible by luminescence and detected by at least one detector.

The term "luminescence" as used below covers all light emissions brought about by a medium given off by the excitation source, and more broadly also emission of ultraviolet and infrared radiation, which emissions are caused not by high temperatures but by prior biological or chemical excitation. Substances

exhibiting luminescence are known as luminophores. As is known to the individual skilled in the art, such luminescence can be elicited by chemical excitation, in which case it is termed chemoluminescence or bioluminescence. The general principles of quantum mechanics underlie this process, which causes excitation of atoms and molecules that then return to their ground state with emission of light, which is detected according to the invention.

The device according to the invention, serving for the chemical excitation of cells for detection of the emitted luminescence signals, comprises in one embodiment a wavelength filter between the surface and the (at least one) detector. Preferably, a plurality of detectors is present, and wavelength filters with different transmission characteristics can be associated with the individual detectors for selective detection of luminescence signals with different wavelengths.

In one embodiment, the carrier element is in the form of a semiconductor body, with an evaluation circuit preferably being integrated into the semiconductor body. The use of an inorganic semiconductor material such as silicon has the advantage that traditional and previously known technology processes such as CMOS processes can be used for manufacturing the carrier with the detectors and the evaluation circuit.

Integration of the evaluation circuit into the semiconductor chip makes it possible to preprocess the detector signals in the immediate vicinity of the cells to be analyzed. Thus, in this preferred embodiment of the present invention, the sensor is a "smart" sensor that is capable of far more than merely passive sensors. For example, the output signals of the electrooptical sensors can be

processed by an integrated circuit so that they can be fed outward without any great difficulty via output circuits and connecting contacts. Also, preprocessing may consist of digitization of the analog sensor signals and their conversion into an appropriate data stream. Moreover, the signal to noise ratio can be very greatly improved by the proximity of the detector to the signal processing location in the device according to the invention, due to the short signal path. In addition, other processing steps are possible, by which for example the data volume can be reduced, or which are used for external processing and display. It is thus possible for the remaining evaluation of the optical signals and their display to be done on a personal computer (PC). Moreover, the device according to the invention can be designed such that the preferably compacted or prepared data can be transmitted by an infrared or radio connection to correspondingly equipped receiver stations.

Furthermore, there is also an option of interest from the cost point of view, of using organic semiconductor materials as described for example in EP-A-1085319 for the carrier with the detectors.

The detectors, located below the surface prepared for receiving cells, are preferably in the form of photodiodes, CCD sensors, or photoconductors. Preferably, a plurality of detectors is integrated matrix-fashion into the carrier in order to carry out a spatially resolved luminescence measurement.

In one embodiment, the excitation source, which is connected to the inlet of the cover, controls, through the evaluation circuit, an excitation medium at the inlet opening to which the cell immobilized at the surface is exposed. For

controlling the supply of medium, for example, a valve is disposed in an inlet line between the excitation source and the inlet opening, said valve being controlled by the control circuit.

In order to bind the cells to the surface of the carrier element, there is the option of placing at the surface an adhesion matrix or a medium such as gelatin that enhances cell adherence and/or cell growth, and allowing the cells actually to grow on this nutrient medium. There is also the option of placing cells on a cell-immobilizing layer, for example negatively charged polystyrene, on the surface.

In one embodiment of the invention, a cell or a cell cluster is immobilized at the surface by the manufacturer, enabling a customer to make measurements on the cell immediately after adding an excitation medium chosen by him or her to the excitation source.

Preferably, the carrier element has several detectors or detector fields, spatially separated from each other, in order to conduct parallel measurements on several cells or cell clusters. These individual detectors or detector fields are preferably disposed separately such that essentially no light emission from a point or field can be picked up by the detector or detectors for another point or field. Thus, the individual detector sites can be disposed in their own depressions, as is known for example with familiar microtiter plates. Preferably, the depressions and the like according to the invention are trough-shaped such that their side walls are essentially perpendicular to the surface of the sensor chip. The dimensions of such a depression may be chosen freely by the individual skilled in the art from knowledge of the application and the depression is preferably at least 100 nm deep in the surface of the carrier element.

Alternatively, vertical partitions pointing upward can be disposed on the essentially planar surface, with their dimensions being at the option of the individual skilled in the art based on knowledge of the application and the spatial dimensions of the cells. Suitable partitions can be applied for example by anodic bonding or by the flip-chip method. Such a device with several detector fields according to the invention makes sensor-supported electrooptical image capturing possible.

In one method according to the invention for detecting cellular processes by detection of luminescence events in, at, or in the immediate vicinity of a cell, a cell cluster, or a tissue, a device is provided having a carrier element with a surface prepared for direct or indirect coupling of cells, at least one optic detector integrated into the carrier element below the surface for receiving a luminescence signal, a cover covering the surface to form a cavity, said cover having an inlet opening and an outlet opening, and an excitation source connected to the inlet opening. At the surface prepared for accepting cells, at least one cell is immobilized, following which a physical or chemical excitation medium is supplied to the cavity from the excitation source, and resulting luminescence events are picked up by the (at least one) detector.

The luminescence events are preferably temporally resolved. This means

that, after an excitation medium has been supplied to the environment of the cell, the light conditions at the detectors are evaluated at multiple sequential points in time, for example at the rate of one per nanosecond.

In order to make metabolic events in the cell visible by luminescence, it is necessary to use luminophores that react with the metabolic products of interest by luminescing, which is picked up by the detectors.

The luminophores suitable for specific applications may be selected in a way familiar to the individual skilled in the art depending on the metabolic product to be detected, with which the luminophore is to react. Luminophores with different half-lives can be differentiated. The half-life indicates the length of time for which luminescence can be measured after excitation. The choice of a luminophore with a half-life appropriate for the particular application is a matter for judgment by the individual skilled in the art. Luminophores whose half-lives are considerably longer than 5 ns, preferably between 100 µs and 2000 µs, are particularly suitable.

The following are suitable in principle: organic luminophores, rare earth metals (lanthanides) or actinide compounds, microspheres (e.g. FluoSpheres® europium luminescent microspheres, molecular probes), or nanocrystals of semiconductors, the latter, in addition to their luminescence properties, being relatively small in size (a few nm) and highly stable (no photobleaching). Other suitable luminophores are alkaline earth halides with lattice defects, which can be made for example by doping (foreign ions) or radioactive radiation.

Most of the above-listed dyes are suitable only for measurements at or in the immediate vicinity of the cells. However, there are methods for rendering cell

membranes permeable to dyes and reporter molecules. Examples of such methods are:

- acetoxymethyl (AM) ester loading

- acid loading (especially for plant cells)

- ATP-induced permeabilization

- cationic liposome delivery

- electroporation

- hypoosmotic shock

- influx pinocytic cell-loading reagent

One appropriate loading system is that described by K. Barber et al.: "Delivery of Membrane-Impermeant Fluorescent Probes into Living Neural Cell Populations by Lipotransfer," *Neurosci. Lett.* 15, 207, 17 (1996).

In one embodiment of the method, the luminescence signal detected by the detector or detectors is compared with a reference value. This reference value can be stored in a memory in the semiconductor body and can be obtained for example by a measurement before excitation of the cell. The reference value can also be obtained in parallel with the measurement at the cell by providing several spatially separated detectors or detector fields, with no cell being immobilized in the region of one detector or detector field, so that detected luminescence signals of this detector or detector field are used as a reference value which takes into account light or radiation influences not caused by the cells to be analyzed, such as the natural fluorescence of system components, and can thus be deducted.

If the sensor surface is designed as a microarray in which a plurality of detector fields each having a number of detectors is present, detection of the measurement field or point signal values can be sequential by detecting whole

rows or columns of the sensor surface or parts thereof one after the other (multiplex application).

The use of multiple parallel detector fields at which the same measurements are carried out at each one also has the advantage that several measurement results are available, from which an overall measurement result can be averaged.

The output signals of the detectors can be evaluated in the semiconductor chip or fed by suitable circuits to an external evaluation device after analog-digital conversion.

It is clear to the individual skilled in the art that the choice of the detector or material depends on the emission wavelength of the dye to be detected. In principle, because of the so-called "semiconductor band gap," the detector has different sensitivities regarding the wavelength of the detected luminescence signal depending on which material is chosen (e.g. silicon or germanium). In the preferred case where a silicon photodiode is used, a sensitivity range from infrared to the ultraviolet wave spectrum is obtained, with the sensitivity being at its greatest between these regions (see for example B. Streetman: "Solid State Electronic Devices," Prentice-Hall, Inc., ISBN 0-13-10 436379-5, pp. 201-227 (1995).

To detect luminescences of different wavelengths, either wavelength-specific photoelements or traditional photodiodes are chosen that are equipped with mechanically applied, vapor-deposited, or integral wavelength filters . For example, it is known that silicon nitride, by contrast to silicon oxide, is impermeable to UV light and that polysilicon absorbs UV radiation (see for

example V.P. Iordanov et al.: "Integrated High Rejection Filter for NADH Fluorescence Measurements," Sensors 2001 Proceedings, Vol. 1, May 8-10, pp. 106-111, AMA Service (2001)). Thus, nitride or polysilicon can be deposited on the gate oxide layer as part of the usual CMOS process, by means of which a corresponding filter is created on the photodiode. Thus, for example, NADH (nicotinamide adenine dinucleotide) has an excitation wavelength of 350 nm and an emission wavelength of 450 nm. When a filter filtering out 350 nm is applied, the sensitivity can be increased.

This effect can be utilized for differential detection when for example two different luminophores, from which for example only a light in the UV range is emitted, are used in parallel, as the detectors provided therefor are made to be UV-sensitive or not. Also, this effect provides the possibility of excluding from the measurement process any materials present that have natural fluorescence that may interfere, and that have known emission wavelengths, by providing the appropriate filters. One example of this is the parallel use of europium chelates (emission at approx. 620 nm) and copper-doped zinc sulfide (emission at approx. 525 nm) that make possible two-color detection because their emission wavelength regions are sufficiently far apart, for example within one region of a detector point or detector field, by for example providing half the sensors of a detector point or detector field with a low-pass filter and the remaining sensors of the same point or field with a high-pass filter.

Additionally or alternatively, different luminophores may be used in parallel as long as their physical or optical properties are sufficiently different. For example, according to the invention the different excitation wavelengths of two luminophores A and B to be used and/or their different half-lives are used.

This can be done for example by providing two differently doped nanocrystals.

The signals of the detectors are received at an evaluation unit. The evaluation unit has a very fast converter to convert analog detector signals into digital values, which are stored. Evaluation of the digital values is preferably done in real time but can also be done on a delayed basis. An ordinary microprocessor can be used for evaluating the digital values. If the luminescence signal is too weak for definitive detection, in a preferred detection embodiment, the detection sensitivity can be heightened by integrating several individual measurements. Here, several identical measurements are carried out and the results are added together. This can be done directly on the sensor chip or after the measurement by appropriate software.

The present invention will now be explained in further detail in exemplary embodiments based on figures:

Figure 1 is a first exemplary embodiment of a device according to the invention for detection of cellular processes by luminescence measurements in a side view in cross section.

Figure 2 is a second exemplary embodiment of a device according to the invention in side view in cross section,

Figure 3 is a third exemplary embodiment of a device according to the invention in side view in cross section, and

Figure 4 is a fourth exemplary embodiment of a device according to the invention in side view in cross section.

In the figures the same reference numerals refer to the same parts with the same significance unless otherwise stated.

Figure 1 shows a first exemplary embodiment of a device according to the invention for detection of a luminescence event in, at, or in the immediate vicinity of a cell, a cell cluster, or a tissue, with only one cell 6 being shown in Figure 1 for clarity. The device has a carrier element 1 with a surface 100 prepared for direct or indirect coupling of cells. For this purpose, the surface includes for example a gelatin layer on which the cell has already grown, or another substance suitable for immobilizing a cell or a cell cluster. Above surface 100 is a cover forming a cavity 70, with the cell being disposed in this cavity. The cover has an inlet 8 and an outlet 9, with the inlet 8 being connected to an excitation source 21 holding an excitation medium.

The device serves for examining the behavior of the cell 6 upon a chemical or biochemical excitation by the excitation medium contained in the excitation source 21, by means of luminescence measurements. The luminescence is created by luminophores that are introduced, in a sufficiently well-known manner, in the cell or in a nutrient solution in the vicinity of the cell, and which react for example with a metabolic product of the cell. The luminescence is detected by detectors 2, for example photodiodes that are integrated below the surface 100 in the carrier element. In the example, a wavelength filter 4 is formed between the surface 100 with the cell 6 and the detectors 2; preferably an optically transparent insulation layer not shown in detail is applied to the detectors 6 to prevent leakage currents across the surface 100, provided the filter 4 is not electrically insulating, is additionally provided with a filter 4*.

---

* Sic – sentence fragment, not beginning with a capital letter. Translator.

A scratch protector 3 with a surface coating 5, for example a precious metal or a hydrophobic/hydrophilic material, is applied to carrier 1, with this scratch protector 3 having a channel cut away above the detectors, at the bottom of which are the filter 4 and the surface 100 prepared for receiving the cell.

An evaluation circuit 11 (shown schematically in Figure 1) is furthermore integrated into the carrier element, for example a semiconductor chip, said circuit being connected to the detectors 2. Connecting lines 21[*] between the evaluation circuit 11 and the detectors 2 are shown only schematically in Figure 1.

The cavity 70 can be supplied from a liquid reservoir 71 with a liquid suitable for storing or washing the cell or keeping it wet. For excitation of the cell, the excitation medium from the excitation source is fed to this liquid, the supply of the excitation medium in the example being controllable by a valve 81 disposed in inlet line 8. This valve 81 is controlled in the example by the integrated evaluation circuit 11.

The excitation medium is chosen for example such that it influences the metabolism of the cell, so that metabolic processes become visible because of the luminophores referred to above and are detected by the detectors 2. Preferably, multiple excitation sources 21 are present and deliver an excitation medium independently of each other so that the behavior of the cell toward different excitation media can be investigated sequentially.

In order to carry away the chemical substances stimulating the cell 6 once the detection process is over, rinsing takes place for example with the liquid from

---

[*] *Sic.* The number "21" has already been used for the excitation source. Translator.

reservoir 71, whereby the medium flows out through outlet 9, or the liquid is suctioned off. The liquid supply from this reservoir is preferably also controlled by a valve 72 that is controlled by the evaluation circuit.

The valve can also be controlled by an external control circuit 12, to which can be fed a signal from evaluation circuit 11 dependent on the output signals of detectors 2, as shown in Figure 2.

Figure 2 shows a second embodiment of a device according to the invention in which two spatially separated detectors 2 are integrated into a carrier 1, for example a semiconductor body 1, below a surface suitable for receiving cells 6. Here, a cell 6 is immobilized above only one of the two detectors 2. The other detector serves to prepare a reference value for the measured value delivered by detector 2 below cell 6. The reference value takes into account any luminescence signals unassociated with the excitation, resulting from natural florescence of the system components for example, and to be deducted from the measurement result. A partition 14 shown in dashed lines preferably projects upward from the carrier 1 to prevent the detector 2, generating the reference value, from being influenced by specific luminescence signals from cell 6 or from the vicinity of the cell.

Figure 3 shows one embodiment of a device according to the invention with a carrier arrangement that differs from both the previous embodiments regarding the arrangement of detectors 2. The embodiment in Figure 3 does not

show the excitation source or the evaluation circuit, which are obviously present as well.

The carrier, preferably formed from a semiconductor chip, has two detector fields 20 in this example, each of which comprises a plurality of detectors. In this way, multiple measurements can be conducted at the same time, so that a statistical evaluation of the measurement signal specifically obtained can be derived. Thus it is possible for example to differentiate between nonspecific and specific signals, the specific signals being the signals resulting from metabolic processes in the cell and the nonspecific signals being other signals, for example interference. These nonspecific signals have a different distribution from the specific signals, making differentiation possible.

Figure 4 shows a variant of a carrier element 2 shown in Figure 3, which has one continuous detector field 20 with a plurality of detectors 2 suitable for examining multiple cells simultaneously. This device makes it possible to examine identical cells under comparable conditions and detect their signals, and these signals obtained under identical conditions can be averaged in an evaluation unit for a statistical check.

A method for manufacturing a carrier suitable for the device according to the invention will be explained briefly below:

A semiconductor chip is manufactured using six-inch wafers by a 0.5 micron CMOS process. The detectors 2 are disposed as pn-photodiodes in an n-well on a p-substrate. After field oxidation, the p-regions of the photodiode are defined and a 10 nm thick gate oxide layer is applied. If desired, a structured

nitride (e.g. LPCVD or PECVD) can additionally be applied at this location as a UV filter. Next, a silicon dioxide layer is applied and structured. After this, the other normal CMOS steps are taken, such as application of a wiring layer and surface passivation (scratch protection).

The CMOS sensor so produced is modified by coating with a suitable cell growth substance such as gelatin. Individual cells (e.g. trypsinized epithelial cells) are seeded and allowed to grow in a cell culture medium (e.g. DMEM-F12).

The device according to the invention is suitable for example for investigating the calcium metabolism of heart muscle cells. The cells are first loaded with aequorin by osmotic shock treatment. Intracellular calcium signals in response to chemical stimuli by the chemical given off by excitation source 21 are made visible by the bioluminescence of the aequorin and detected by the detectors.

The device according to the invention for investigating intracellular processes after biological or chemical excitation is particularly compact and can be made by known methods.